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Inheritance of Resistance of Bovine Preimplantation Embryos to Heat Shock: Relative Importance of the Maternal Versus Paternal Contribution

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ABSTRACT Brahman preimplantation embryos are less affected by exposure to heat shock than Holstein embryos. Two experiments were conducted to test whether the ability of Brahman embryos to resist the deleterious effects of heat shock was a result of the genetic and cellular contributions from the oocyte, spermatozoa, or a combination of both. In the first experiment, Brahman and Holstein oocytes were collected from slaughterhouse ovaries and fertilized with spermatozoa from an Angus bull. A different bull was used for each replicate to eliminate bull effects. On day 4 after fertilization, embryos ≥ 9 cells were collected and randomly assigned to control (38.5°C) or heat shock (41°C for 6 hr) treatments. The proportion of embryos developing to the blastocyst (BL) and advanced blastocyst (ABL; expanded and hatched) stages was recorded on day 8. Heat shock reduced the number of embryos produced from Holstein oocytes that developed to BL ($P < 0.001$, $55.6 \pm 4.2\%$ vs. $29.8 \pm 4.2\%$) and ABL ($P < 0.01$, $37.7 \pm 3.6\%$ vs. $12.2 \pm 3.6\%$) on day 8 as compared to controls. In contrast, heat shock did not reduce development of embryos produced from Brahman oocytes (BL = $42.1 \pm 4.8\%$ vs. $55.6 \pm 4.8\%$ for 38.5 and 41°C, respectively; ABL = $17.6 \pm 4.2\%$ vs. $32.4 \pm 4.2\%$). In the second experiment, oocytes from Holstein cows were fertilized with semen from bulls of either Brahman or Angus breeds. Heat shock of embryos ≥ 9 cells reduced development to BL ($P < 0.002$) and ABL ($P < 0.005$) for embryos sired by both Brahman (BL = $54.3 \pm 7.7\%$ vs. $23.4 \pm 7.7\%$; ABL = $43. \pm 7.4\%$ vs. $7.9 \pm 7.4\%$, for 38.5 and 41°C, respectively) and Angus bulls (BL = $57.9 \pm 7.7\%$ vs. $31.0 \pm 7.7\%$; ABL = $33.6 \pm 7.4\%$ vs. $18.4 \pm 7.4\%$, for 38.5 and 41°C, respectively). There were no breed × temperature interactions. Results suggest that the oocyte plays a more significant role in the resistance of Brahman embryos to the deleterious effects of heat shock than the spermatozoa. *Mol. Reprod. Dev. 63: 32–37, 2002.*

Key Words: heat shock; embryo; cattle breed

INTRODUCTION

Heat stress in cattle has an adverse impact on multiple physiological functions important to reproduction. Heat stress reduces uterine blood flow (Roman-Ponce et al., 1978), alters the secretion of reproductive hormones and follicular growth dynamics (Roman-Ponce et al., 1981; Badinga et al., 1994; Wolfenson et al., 1995; Trout et al., 1998), compromises oocyte competence (Rocha et al., 1998; Zeron et al., 2001; Al-Katanani et al., 2002), and reduces embryonic development (Putney et al., 1988; Ealy et al., 1993). While heat stress adversely affects reproduction in cattle, *Bos indicus* and certain tropically-adapted *Bos taurus* breeds are less affected by exposure to high ambient temperature and humidity than *Bos taurus* cattle developed in Europe. The ability of the *Bos indicus* to more effectively regulate body temperature during hyperthermia than *Bos taurus* breeds (Cartwright, 1955; Finch, 1986; Carvalho et al., 1995; Hammond et al., 1996) means that reproductive function is less compromised in tropical and sub-tropical regions (Turner, 1982). Rocha et al. (1998) reported that high temperature and humidity was associated with a significant decline in the quality of oocytes recovered from *Bos taurus* cows (Holstein and crossbred Angus) and their in vitro development following fertilization. In contrast, oocytes retrieved from Brahman cows displayed normal morphology and their development in vitro was not compromised.

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The superior reproductive function of breeds such as the Brahman during heat stress may also be due, at least in part, to increased resistance to heat shock at the cellular level. Oviductal and endometrial tissue from Brahman cows responded differently to heat shock than the same tissue from Holstein cows (Malayer and Hansen, 1990). Furthermore, Brahman lymphocytes were more resistant to the lethal effects of heat shock than Angus lymphocytes (Kamwanja et al., 1994). In a recent study (Paula-Lopes et al., 2001), Brahman preimplantation embryos were less affected by heat shock (41°C for 6 hr) than embryos from both Holstein and Angus cows.

It is not known whether contributions of the spermatozoa or the oocyte are more important for embryonic thermotolerance. If spermatozoa contribute genetic or nonnuclear factors which are necessary to resist the deleterious effects of heat shock, then it may be possible to use semen from thermotolerant bulls in artificial insemination to improve the fertility of thermosensitive animals during heat stress. Furthermore, identifying whether resistance to heat shock is due to a maternal or paternal factor may make it easier to identify genes or other cytoplasmic components responsible for cellular resistance to heat shock. The objective of the present study was to determine whether the ability of Brahman embryos to resist the deleterious effects of heat shock is due primarily to contributions of the spermatozoa, the oocyte, or a combination of both.

MATERIALS AND METHODS

Materials

The media SP-TL, IVF-TL, and HEPES-TL were purchased from Cell and Molecular Technologies, Inc. (Lavallete, NJ) and used to prepare Sperm-Tyrode's albumin lactate pyruvate (TALP), IVF-TALP, and HEPES-TALP as previously described (Parrish et al., 1986). Potassium simplex optimized medium (KSOM) was also obtained from Cell and Molecular Technologies. Bovine steer serum was purchased from Pel-Freez (Rogers, AR) and Percoll from Amersham Pharmacia Biotech (Uppsala, Sweden). Follicle stimulating hormone was Folltropin®-V from Vetrepharm Canada (London, Ont.) and was purchased from Agtech (Manhattan, KS). Polyvinylpyrrolidone (PVP) was obtained from Eastman Kodak (Rochester, NY). Paraformaldehyde was from Fisher Scientific (Fair Lawn, NJ) and Prolong® Antifade kit was purchased from Molecular Probes (Eugene, OR). Hoechst 33258 dye was from Sigma (St. Louis, MO). All other chemicals were obtained from Sigma or Fisher Scientific.

Production of Embryos

Production of embryos using in vitro maturation, fertilization, and embryo culture was performed as previously described (Rivera and Hansen, 2001). A detailed procedure can be found at http://www.animal.ufl.edu/hansen/IVF. Briefly, ovaries from Holstein and Brahman cows were obtained from a local abattoir (approximately 1.5 hr from the laboratory) and transported to the laboratory in 0.9% (w/v) NaCl at room temperature or were obtained from the University of Florida abattoir. The ovaries were sliced and cumulus-oocyte complexes (COCs) were collected into a beaker containing oocyte collection medium [Tissue Culture Medium-199 with Hank's salts without phenol red and supplemented with 2% (w/v) bovine steer serum (100 IU penicillin ml^{-1} and 0.1 mg streptomycin ml^{-1}]. The COCs were cultured in 50 μl drops of oocyte maturation medium [Tissue Culture Medium-199 with Earle's salts supplemented with 10% (v/v) bovine steer serum, 100 IU penicillin ml^{-1}, 0.01 mg streptomycin ml^{-1}, 2 μg estradiol ml^{-1}, 20 μg FSH ml^{-1}, and 0.2 mmol sodium pyruvate l^{-1}] in groups of 10 for 22–24 hr. Following maturation, the COCs were washed in HEPES-TALP and then placed in groups of approximately 30 in 600 μl IVF-TALP in four-well plates. Spermatozoa were purified by Percoll gradient (Parrish et al., 1986), suspended in SP-TALP, and then added to matured oocytes at a density of $\sim 1 \times 10^6$ spermatozoa/well. After 8–10 hr, putative zygotes were harvested and denuded of cumulus cells by vortexing for 5 min in a 2.0 ml microcentrifuge tube containing 0.5 ml HEPES-TL. Putative zygotes were cultured in 50 μl drops of modified KSOM until day 4 after fertilization. All cultures were carried out at 38.5°C in 5% CO_2 in humidified air unless specified otherwise.

Experiment 1: Breed of Oocyte Effects on Resistance of Bovine Embryos to Heat Shock

Holstein and Brahman oocytes ($n = 366$ and 300, respectively) were collected as described above and fertilized using spermatozoa from an Angus bull. A different Angus bull was used for each replicate ($n = 8$ replicates for Holstein and $n = 6$ replicates for Brahman) to eliminate bull effects. On day 4 following fertilization (fertilization = day 0), embryos ≥ 9 cell from each breed of oocyte were separated, placed into microdrops of modified KSOM, and randomly assigned to one of the two treatments: control or heat shock. Embryos assigned to the control treatment were placed in an incubator at 38.5°C and 5% CO_2 until day 8. Embryos in the heat shock group were placed in an incubator at 41°C for 6 hr. The incubator contained 7% CO_2 in humidified air to maintain the pH of the culture medium. After 6 hr of heat shock, embryos were returned to 38.5°C and 5% CO_2 until day 8. The embryos were divided so that an equal number of embryos per drop existed across breed and treatment within a given replicate. For groups of five embryos or less, embryos were placed in 10 μl drops of modified KSOM. For groups greater than five, embryos were placed in 25 μl drops of modified KSOM. On day 8 after fertilization, the proportion of embryos developing to the blastocyst (BL) and advanced blastocyst (ABL; expanded, hatching, and hatched) stages was recorded.

Experiment 2: Breed of Sire Effects on Resistance of Bovine Embryos to Heat Shock

Holstein oocytes were collected at a local abattoir. Following maturation, approximately half were fertilized using spermatozoa from an Angus bull while half were fertilized using spermatozoa from a Brahman bull. The experiment was replicated seven times with a total of 309 (Angus) and 324 (Brahman) oocytes. A different bull was used for each replicate. Embryos ≥ 9 cells were separated on day 4 as for experiment 1 and placed into fresh culture drops. Embryos were either cultured at 38.5°C continuously or placed at 41°C and 7% CO_2 for 6 hr and then returned to 38.5°C. The proportion of embryos that developed to BLs and ABLs (expanded, hatching, and hatched) was recorded on day 8 after fertilization.

Embryo Cell Number

Two different staining protocols were used. For eight replicates of Experiment 1, a slightly modified version of the protocol described by Pursel et al. (1985) was used. Briefly, embryos that developed to the BL stage were placed on slides and covered with 0.01% (w/v) Trypan Blue dye for 30–60 sec at room temperature. The Trypan Blue dye was then removed and Hoechst 33258 dye (10 μg/ml) was added for 5 min at 38.0°C. The Hoechst dye was removed and coverslips were mounted with Prolong Antifade mounting medium. Coverslips were firmly pushed on the slide to separate the embryo. For six replicates of Experiment 1 and all of Experiment 2, cell number was determined using Hoechst 33258 dye. Embryos were washed in PBS containing 1 mg/ml polyvinylpyrrolidone and then placed in 4% (v/v) paraformaldehyde in PBS for 1 hr. Embryos were washed, fixed to poly-L-lysine coated slides and stored in a slide box at 4°C until staining. For staining, embryos were covered with 1 μg/ml Hoechst 33258 for 5 min at room temperature. Hoechst dye was then removed by blotting and coverslips were mounted using Prolong Antifade mounting medium. For both experiments, fluorescent staining of nuclei was visualized with a Zeiss Axiovert 135 epifluorescence microscope 2 hr after coverslips had been mounted.

Statistical Analysis

Data collected were the percentage of oocytes reaching a certain stage at day 4 after insemination (cleaved, 4–8 cell, ≥ 9 cell) and the percentage of embryos developing to the BL and ABL stage at day 8 after insemination. Percent development was calculated for each replicate. Treatment effects were analyzed by least squares ANOVA using the GLM procedure of SAS (SAS Institute, 1989). For both experiments, replicate (i.e., bull used for insemination) was considered a random effect and other main effects were considered fixed. Tests of significance were performed using calculated expected mean squares. Percentage data were transformed by arcsine transformation before analysis. Probability values are reported from analysis of transformed data while least squares means ± SEM are derived from analyses of untransformed data.

RESULTS

Experiment 1: Breed of Oocyte Effects on Resistance of Bovine Embryos to Heat Shock

At day 4 after insemination, there was no effect of breed of oocyte on cleavage rate. In addition, there was no effect of breed on the proportion of oocytes or the proportion of cleaved embryos that were at the < 4 cell, 4–8 cell, and ≥ 9 cell stages (Table 1).

For embryos at the ≥ 9 cell stage on day 4 after insemination, there were breed × temperature interactions affecting development to the BL stage ($P < 0.001$) and ABL stage ($P < 0.01$). Exposure of embryos from Holstein oocytes to heat shock reduced development as compared to that for embryos cultured at 38.5°C. In contrast, heat shock did not decrease development of embryos from Brahman oocytes. In fact, development was greater for 41°C (Fig. 1).

Heat shock did not significantly reduce BL cell number and there was no breed × temperature interaction. However, there was a trend for BLs developed from Brahman oocytes to have a higher cell number

TABLE 1. Cleavage Rate and Stage of Embryonic Development at Day 4 After In Vitro Insemination for Brahman and Holstein Oocytes Fertilized With Angus Spermatozoa

	Brahman	Holstein
Cleavage rate (%)[a]	83.0 ± 4.3% (246/300)	76.9 ± 3.7% (282/366)
Percent of total oocytes		
2–3 cell	10.4 ± 2.4% (32/300)	8.3 ± 2.1% (31/366)
4–8 cell	32.1 ± 3.0% (95/300)	25.4 ± 2.6% (91/366)
≥ 9 cell	40.5 ± 5.3% (119/300)	43.2 ± 4.6% (160/366)
Percent of cleaved oocytes		
2–3 cell	13.4 ± 4.2% (32/246)	11.6 ± 3.6% (31/282)
4–8 cell	39.1 ± 3.3% (95/246)	32.9 ± 2.8% (91/282)
≥ 9 cell	47.5 ± 4.6% (119/246)	55.5 ± 4.0% (160/282)

[a]Data represent the least-squares means ± SEM. Data within parentheses represent the proportion of oocytes across all experimental replicates.

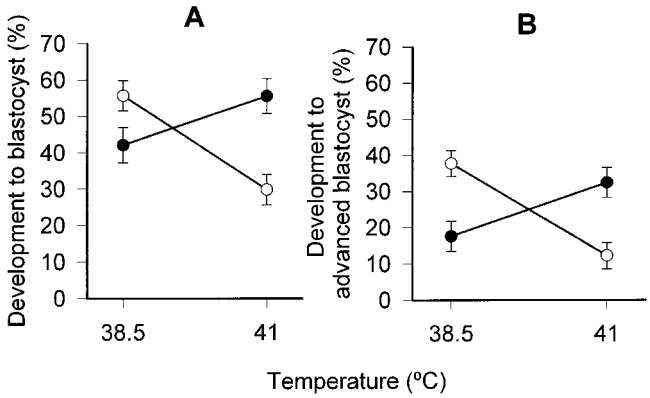

Fig. 1. Effect of heat shock on development of day 4 embryos (≥ 9 cells) produced from Brahman oocytes (●) and Holstein oocytes (○). **Panel A** represents the proportion of embryos developing to BL at day 8 after insemination. **Panel B** represents the proportion of embryos that developed into ABLs (expanded, hatching, or hatched) on day 8. Data are least-squares means ± SEM. There was a breed × temperature interaction affecting percent BL ($P < 0.001$) and percent ABL ($P < 0.005$).

than those developed from Holstein oocytes ($P < 0.09$, Fig. 2).

Experiment 2: Breed of Sire Effects on Resistance on Bovine Embryos to Heat Shock

There was no effect of breed of sire on the percentage of oocytes that had cleaved on day 4 after insemination. The proportion of oocytes that were < 4 cell stage embryos on day 4 tended ($P < 0.07$) to be lower for Brahman-sired embryos. Moreover, the proportion of cleaved embryos at the < 4 cell stage on day 4 was significantly higher for Brahman-sired embryos ($P < 0.02$). There was no difference between the two breeds of sire

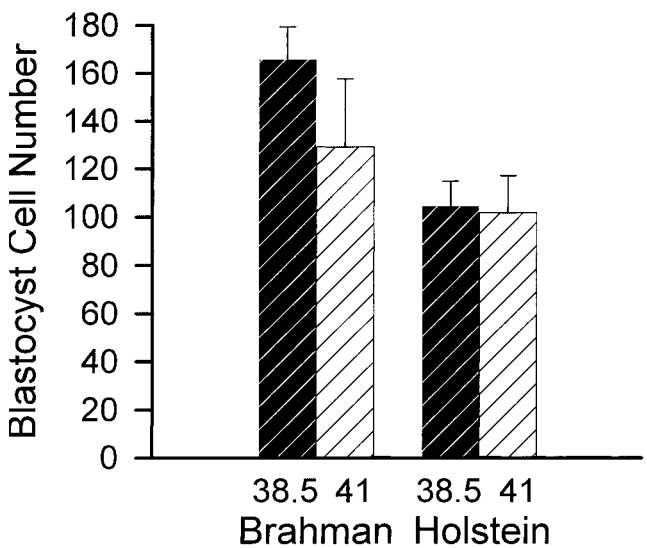

Fig. 2. BL cell number on day 8 as affected by heat shock at the ≥ 9-cell stage and breed of oocyte (Brahman and Holstein). Main effects and interactions were not significant. Data represent least-squares means ± SEM for 9 (Brahman control), 2 (Brahman heat shock), 16 (Holstein control), and 7 (Holstein heat shock) embryos.

for the proportion of oocytes or cleaved embryos at the 4–8 cell and ≥ 9 cell stages on day 4 after insemination (Table 2).

For embryos that were ≥ 9 cell, heat shock significantly reduced the percentage of embryos developing to the BL ($P < 0.002$,) and ABL stage ($P < 0.005$, Fig. 3). The reduction caused by heat shock was similar for embryos of both breeds of sire and there was no breed × temperature interaction. BL cell number was not significantly affected by any main effect or interaction, although there was a trend ($P < 0.07$) for heat shock to reduce BL cell number on day 8 (Fig. 4).

DISCUSSION

The superior thermoregulatory ability of the Brahman breed of cattle compared to most *Bos taurus* breeds is well known (Cartwright, 1955; Finch, 1986; Carvalho et al., 1995; Hammond et al., 1996). Differences between Brahman and *Bos taurus* breeds of cattle in response to heat shock at the cellular level also have been observed (Malayer and Hansen, 1990, Kamwanja et al., 1994). Most recently, Paula-Lopes et al. (2001) reported that Brahman preimplantation embryos were less affected by exposure to heat shock (41°C for 6 hr) than embryos from both Holstein and Angus breeds. The primary objective of the present study was to determine whether the ability of Brahman embryos to resist the deleterious effects of heat shock was due to contributions of the oocyte, the spermatozoa, or both. Results indicate that the contribution of the oocyte plays a more crucial role in the ability of Brahman embryos to resist effects of heat shock than the contribution of the spermatozoa. Embryos at the ≥ 9 cell stage at day 4 after fertilization (i.e., the normal stage of development) were more affected by heat shock if produced using Holstein oocytes than if using Brahman oocytes. In contrast, breed of sire had no effect on the thermal resistance of embryos produced using Angus and Brahman semen. These results have relevance for understanding cytoprotective responses in the preimplantation embryo and for practical approaches to increase pregnancy rate in heat-stressed cows.

There are several putative explanations for the importance of the oocyte versus the sperm for determining cellular resistance of embryos to heat shock. One possibility is that the oocyte has cytoplasmic factors that persist into embryonic life that allow increased resistance to heat shock. These factors could include mitochondria, mRNA, antioxidants or proteins. Most of the cytoplasmic inheritance in mammals comes from the oocyte (Smith and Alcivar, 1993). A portion of maternal mRNA transcripts are still present in the mouse BL (Duranthon and Renard, 2001), so it is possible that the differences observed in this study are due to differences between Brahman and Holstein oocytes in the levels of maternal mRNA for one or more cytoprotective proteins. One such molecule is heat shock protein 70 (HSP70), which functions during heat shock by serving as a molecular chaperone and anti-apoptotic molecule (Hansen, 1999). However, Brahman

TABLE 2. Cleavage Rate and Development of Holstein Oocytes on Day 4 After In Vitro Fertilization With Brahman and Angus Spermatozoa

	Brahman	Angus
Cleavage rate (%)[a]	63.6 ± 7.5% (203/324)	77.0 ± 7.5% (230/309)
Percent of Total Oocytes		
2–3 cell	8.8 ± 1.3% (28/324)	5.1 ± 1.3% (15/309)
4–8 cell	14.9 ± 2.8% (48/324)	21.8 ± 2.8% (64/309)
≥ 9 cell	39.3 ± 6.8% (125/324)	50.1 ± 6.8% (159/309)
Percent of Cleaved Oocytes		
2–3 cell[b]	14.4 ± 1.9% (28/203)	6.7 ± 1.9% (15/230)
4–8 cell	25.5 ± 4.1% (48/203)	28.4 ± 4.1% (64/230)
≥ 9 cell	59.5 ± 5.3% (125/203)	64.9 ± 5.3% (159/230)

[a]Data represent the least-squares means ± SEM. Data within parentheses represent the proportion of oocytes across all experimental replicates.
[b]$P < 0.02$.

lymphocytes exposed to heat shock did not produce greater amounts of HSP68 than lymphocytes from Senepol and Angus, even though a larger proportion of Brahman lymphocytes were viable after heat shock than lymphocytes from the thermosensitive Angus breed (Kamwanja et al., 1994). Perhaps non-nuclear components such as the mitochondria and cellular membranes may be less affected by heat shock for an embryo derived from a Brahman oocyte than for an embryo derived from a Holstein oocyte. Chandolia et al. (1999) reported that there were no significant differences between Brahmans and Holsteins in responses of bovine spermatozoa to heat shock. Since the sperm is transcriptionally inactive, this result argues against important differences in at least the non-nuclear components present in sperm (mitochondria and the plasma membrane).

Paula-Lopes et al. (2001) also noted differences between Brahman embryos and other breeds in thermoresistance at the ≥ 9-cell stage. There were, however, no breed differences in response to heat shock for embryos at the 2-cell stage (Krininger et al., 2001). Given that major genome activation occurs at the 8-cell stage (Memili and First, 2000), these observations imply that the capacity for embryonic transcription is a requirement for breed differences. If so, the failure of breed of sire to affect embryonic resistance to heat shock implies that the thermoprotective genes are paternally imprinted. Lyle (1997) reported the existence of 21 imprinted genes, the majority of which have been identified in both humans and mice. To our knowledge, no genomic imprinted gene that confers thermotolerance has been identified to date.

Exposure to heat shock did not reduce BL cell number in either experiment. Although a lower percentage of embryos produced from Holstein oocytes (regardless of breed of sire) reached the BL and ABL stages after heat shock compared with Brahman oocyte-derived embryos, the embryos that reached these developmental stages did not have significantly fewer cells than

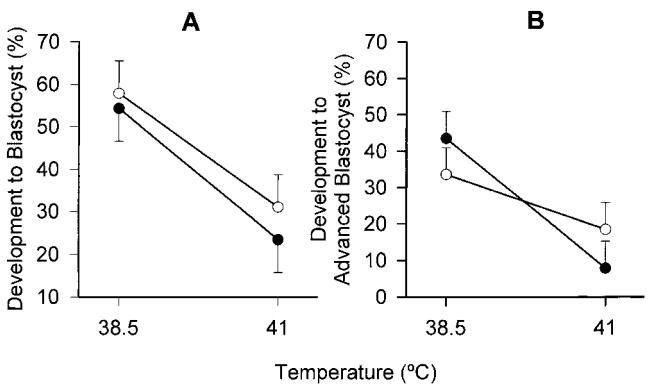

Fig. 3. Effects of heat shock on development of day 4 embryos (≥ 9 cells) fertilized with Brahman (●) and Angus (○) spermatozoa. **Panel A** represents the proportion of embryos developing to BL at day 8 after insemination. **Panel B** represents the proportion of embryos that developed into ABLs (expanded, hatching, or hatched) on day 8. Data are least-squares means ± SEM. There was a significant effect of temperature affecting percent BL ($P < 0.002$) and percent ABL ($P < 0.005$).

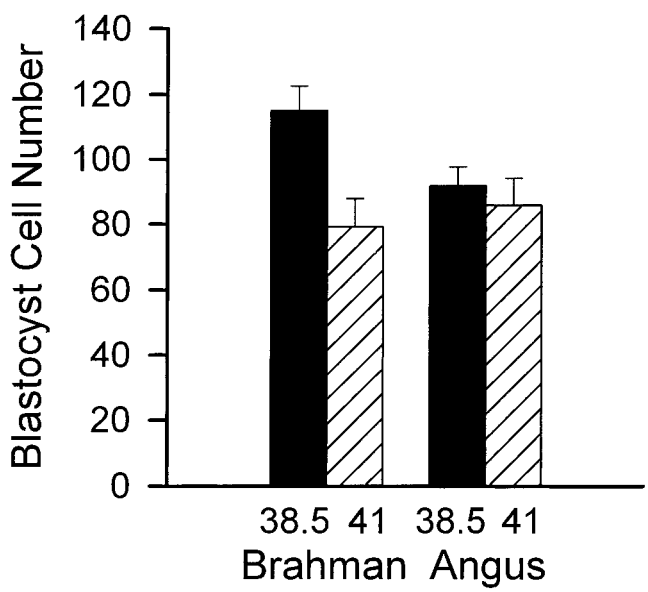

Fig. 4. BL cell number on day 8 as affected by heat shock at the ≥ 9 cell stage and breed of sire (Brahman and Angus). Main effects and interactions were not significant. Data represent least-squares means ± SEM for 21 (Brahman control), 16 (Brahman heat shock), 34 (Angus control), and 17 (Angus heat shock) embryos.

control embryos. These results suggest that an embryo which escapes the effects of heat shock is a normal one. However, other aspects of blastomere function could be compromised. For example, it has been reported that heat shock increases the percentage of blastomeres that undergo apoptosis. Exposure of ≥ 16 cell embryos to heat shock on day 5 after insemination increased the percentage of blastomeres that underwent apoptosis and this was also observed for 8–16 cell embryos on day 4 after insemination (Paula-Lopes and Hansen, 2000). The percentage of apoptotic cells within embryos on day 8, however, was not looked at in these experiments.

Although the findings of this study indicate that the use of artificial insemination with semen from thermotolerant breeds would not increase pregnancy rates in heat stressed cattle, it is possible that genes which confer thermotolerance can be identified. Once genes that are responsible for cellular resistance to heat shock are found, it would be possible to introduce such genes into thermosensitive populations of cattle, which may improve production of these animals under heat stress conditions.

In conclusion, the results of this study indicate that the contribution of the oocyte is more important than that of the sperm for conferring thermotolerance in bovine embryos. Identification of the mechanism by which breed confers thermoresistance may lead to the discovery of genes that exist in Brahman cattle that confer cellular thermotolerance. In addition, the possible role of imprinting of thermoresistance genes may lead to identification of additional imprinted genes.

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